

METHOD OF PRODUCTION OF RECOMBINANT SUCROSE SYNTHASE,  
USE THEREOF IN THE MANUFACTURE OF KITS FOR  
DETERMINATION OF SUCROSE, PRODUCTION OF ADPGLUCOSE AND  
PRODUCTION OF TRANSGENIC PLANTS WHOSE LEAVES AND  
5 STORAGE ORGANS ACCUMULATE HIGH CONTENTS OF ADPGLUCOSE  
AND STARCH

AREA OF INDUSTRY TO WHICH THE INVENTION RELATES

10 The invention relates to optimization of the production  
of recombinant sucrose synthase (SS) in soluble, active  
form employing an appropriate strain of *Escherichia*  
*coli*, the use of SS for making kits for determination  
of sucrose, design of optimized forms of SS for the  
15 synthesis of ADPglucose (ADPG), and the production of  
transgenic plants whose leaves and storage tissues  
accumulate high levels of ADPG and amylose-enriched  
starch as a result of overproduction of cytosolic ADPG  
in plants which overexpress SS.

20

PRIOR ART

Starch is the main storage form of carbohydrates in  
plants. It accumulates in large amounts in organs such  
25 as seeds (wheat, barley, maize, pea, etc.) and tubers  
(potato and yam among others) and is a fundamental  
constituent of the human diet. Furthermore, starch is  
widely used in the paper, cosmetic, pharmaceutical and  
food industries, and is also used as an essential  
30 component for the manufacture of biodegradable plastics  
and environment-friendly paints. Since it is made up of  
covalently bound glucose molecules, investigation of  
the processes involved in the synthesis of this  
polysaccharide is a top priority in various areas of  
35 industrial production.

ADPG is the universal precursor of starch biosynthesis  
in plants, both in heterotrophic organs (Fig. 1A) and  
in leaves (Fig. 2A), and it is widely assumed that its

production is controlled exclusively by the enzyme ADPG  
pyrophosphorylase (AGPase) or ADPG synthase (EC  
2.7.7.27) (Okita, T.W. (1992) Is there an alternative  
pathway for starch synthesis? Plant Physiol. 100, 560-  
5 56; Müller-Röber, B., Sonnewald, U. Willmitzer, L.  
(1992) Inhibition of the ADPglucose pyrophosphorylase  
in transgenic potatoes leads to sugar-storing tubers  
and influences tuber formation and expression of tuber  
storage protein genes. EMBO J. 11, 1229-1238; Stark,  
10 D.M., Timmerman, K.P., Barry, G.F., Preiss, J.,  
Kishore, G.M. (1992) Regulation of the amount of starch  
in plant tissues by ADPglucose pyrophosphorylase.  
Science 258, 287-282; Neuhaus, E.H., Häusler, R.E.,  
Sonnewald, U. (2005) No time to shift the paradigm on  
15 the metabolic pathway to transitory starch in leaves.  
Trends Plant Sci. at press). The various applications  
of the starch produced in a plant are based mainly on  
the ratio of amylose and amylopectin, which determines  
the structure of the starch grain, as well as its  
20 viscosity in aqueous suspensions. This ratio of amylose  
and amylopectin depends on, among other things, the  
concentration of ADPG in the plant cell (Clarke, B.R.,  
Denyer, K., Jenner, C.F., Smith, A.M. (1999) The  
relationship between the rate of starch synthesis, the  
25 adenosine 5'-diphosphoglucose concentration and the  
amylose content of starch in developing pea embryos.  
Planta 209, 324-329).

SS (EC 2.4.1.13, SS) (UDP-glucose:D-fructose-2-glucosyl  
30 transferase) is a reversible enzyme that catalyses the  
production of UDPG and fructose from sucrose and UDP.  
Although, as shown in Fig. 1A, SS has classically been  
regarded as having the role of producing UDPG,  
metabolic processing of which eventually gives rise to  
35 the production of starch in heterotrophic tissues such  
as endosperm and tubers (Zrenner, R., Salanoubat, M.,  
Willmitzer, L., Sonnewald, U. (1995) Evidence for the  
crucial role of sucrose synthase for sink strength  
using transgenic potato plants. Plant J. 7, 97-107;

Baroja-Fernández, E., Muñoz, F.J., Saikusa, T.,  
Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J.  
(2003) Sucrose synthase catalyzes the de novo  
production of ADPglucose linked to starch biosynthesis  
5 in heterotrophic tissues of plants. *Plant Cell Physiol.*  
44, 500-509; Pozueta-Romero, J., Muñoz, F.J.,  
Rodríguez-López, M., Baroja-Fernández, E., Akazawa, T.  
(August 2003) New waves in the starch field. *Lett.*  
*Plant Cell Physiol.* 24-32), there are references to the  
10 potential ability of the enzyme to use other nucleotide  
diphosphates in vitro for the production of the  
corresponding sugar nucleotides (Murata, T., Sugiyama,  
T., Minamikawa, T., Akazawa, T. (1966) Enzymic  
mechanism of starch synthesis in ripening rice grains.  
15 *Mechanism of the sucrose-starch conversion. Arch.*  
*Biochem. Biophys.* 113, 34-44; Delmer, D.P. (1972) The  
purification and properties of sucrose synthase from  
etiolated *Phaseolus aureus* seedlings. *J. Biol. Chem.*  
247, 3822-3828). Although of questionable physiological  
20 relevance (Okita, T.W. (1992) Is there an alternative  
pathway for starch synthesis? *Plant Physiol.* 100, 560-  
56; Müller-Röber, B., Sonnewald, U. Willmitzer, L.  
(1992) Inhibition of the ADPglucose pyrophosphorylase  
in transgenic potatoes leads to sugar-storing tubers  
25 and influences tuber formation and expression of tuber  
storage protein genes. *EMBO J.* 11, 1229-1238), it has  
been suggested that SS is capable of producing ADPG  
directly, which can be used for the production of  
starch both in heterotrophic tissues and in  
30 photosynthetic tissues (Figs. 1B and 2B) (Pozueta-  
Romero, J., Perata, P., Akazawa, T. (1999) Sucrose-  
starch conversion in heterotrophic tissues of plants.  
*Crit. Rev. Plant Sci.* 18, 489-525; Baroja-Fernández,  
E., Muñoz, F.J., Akazawa, T., Pozueta-Romero, J. (2001)  
35 Reappraisal of the currently prevailing model of starch  
biosynthesis in photosynthetic tissues: a proposal  
involving the cytosolic production of ADPglucose by  
sucrose synthase and occurrence of cyclic turnover of  
starch in the chloroplast. *Plant Cell Physiol.* 42,

1311-1320; Baroja-Fernández, E., Muñoz, F.J., Saikusa, T., Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J. (2003) Sucrose synthase catalyzes the de novo production of ADPglucose linked to starch biosynthesis in heterotrophic tissues of plants. Plant Cell Physiol. 44, 500-509; Baroja-Fernández, E., Muñoz, F.J., Zanduetta-Criado, A., Morán-Zorzano, M.T., Viale, A.M., Alonso-Casajús, N., Pozueta-Romero, J. (2004) Most of ADPglucose linked to starch biosynthesis occurs outside the chloroplast in source leaves. Proc. Natl. Acad. Sci. USA 101, 13080-13085). According to this hypothesis (based solely and circumstantially on evidence of the biochemical type), SS is responsible for the synthesis of an important pool of ADPG molecules necessary for the biosynthesis of starch. This hypothesis has not, however, been demonstrated experimentally by genetic engineering or traditional crop improvement techniques, and is not consistent with the countless tests of the genetic and molecular type indicating that AGPase is the only source of ADPG in plants (Okita, T.W. (1992) Is there an alternative pathway for starch synthesis? Plant Physiol. 100, 560-566; Müller-Röber, B., Sonnewald, U. Willmitzer, L. (1992) Inhibition of the ADPglucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11, 1229-1238; Neuhaus, E.H., Häusler, R.E., Sonnewald, U. (2005) No time to shift the paradigm on the metabolic pathway to transitory starch in leaves. Trends Plant Sci. at press).

Sugar nucleotides such as UDPG and ADPG are produced commercially from pyrophosphorylase reactions catalysed by enzymes such as UDPG pyrophosphorylase (UGPase) and AGPase, respectively, based on the use of an expensive substance called glucose-1-phosphate (G1P). An alternative to this practice for production of sugar nucleotides is based on the use of SS, development of

which has largely been hampered by the limitations of *Escherichia coli* for expressing and efficiently processing a large number of eukaryotic proteins. This limitation inspired some researchers to produce

5 recombinant SS by making use of biological factories of the eukaryotic type such as yeasts (Zervosen, A., Römer, U., Elling, L. (1998) Application of recombinant sucrose synthase-large scale synthesis of ADP-glucose. J. Mol. Catalysis B: Enzymatic 5, 25-28; Römer, U.,

10 Schrader, H., Günther, N., Nettelstroth, N., Frommer, W.B., Elling, L. (2004) Expression, purification and characterization of recombinant sucrose synthase I from *Solanum tuberosum* L. for carbohydrate engineering. J. Biotechnology 107, 135-149). Alternatively, SS intended

15 for the production of sugar nucleotides has had to be purified by expensive processes of purification of proteins from plant extracts (patent DE4221595 (1993), Purified sucrose synthase enzyme useful for production of nucleotide-activated sugars or oligosaccharides).

20 This SS obtained from plant extracts has the disadvantage that it has a predilection for UDP and very low affinity for ADP (Pressey R (1969) Potato sucrose synthase: purification, properties, and changes in activity associated with maturation. Plant Physiol.

25 44, 759-764; Nguyen-Quock, B., Krivitzky, M., Huber, S.C., Lecharny, A. (1990) Sucrose synthase in developing maize leaves. Plant Physiol. 94, 516-523; Morell, M., Copeland, L. (1985) Sucrose synthase of soybean nodules. Plant Physiol. 78, 149-154).

30 Production of recombinant SS from cultures of *E. coli* has recently been achieved (Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F., Hayashi, T. (1997) "Expression and characterization of sucrose synthase from mung bean seedlings in *Escherichia coli*" Biosci.

35 Biotech. Biochem. 61, 1500-1503; Nakai, T., Konishi, T., Zhang, Z-Q., Chollet, R., Tonouchi, N., Tsuchida, T., Yoshinaga, F., Mori, H., Sakai, F., Hayashi, T. (1997) "An increase in apparent affinity for sucrose of mung bean sucrose synthase is caused by in vitro

phosphorylation or directed mutagenesis of Ser11" Plant Cell Physiol. 39, 1337-1341; Barratt, D.H.P., Barber, L., Kruger, N.J., Smith, A.M., Wang, T.L., Martin, C. (2001) Multiple, distinct isoforms of sucrose synthase  
5 in pea. Plant Physiol. 127, 655-664; Christopher, B., William, B., Robert, H. "Bacterial sucrose synthase compositions and methods of use" Patent WO9803637). However, the production of SS in this prokaryotic system was associated with problems such as (1) the  
10 amount of SS produced was very low (30 micrograms/gram of bacteria, Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F., Hayashi, T. (1997) "Expression and characterization of sucrose synthase from mung bean seedlings in *Escherichia coli*" Biosci. Biotech.  
15 Biochem. 61, 1500-1503; Li, C.R., Zhang, X.B., Hew, C.S. (2003) "Cloning, characterization and expression analysis of a sucrose synthase gene from tropical epiphytic orchid *Oncidium goldiana*. Physiol. Plantarum 118, 352-360), (2) the amount of active SS obtained was  
20 very low or zero (0.05-1.5 units/mg (Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F., Hayashi, T. (1997) "Expression and characterization of sucrose synthase from mung bean seedlings in *Escherichia coli*" Biosci. Biotech. Biochem. 61, 1500-  
25 1503; Li, C.R., Zhang, X.B., Hew, C.S. (2003) "Cloning, characterization and expression analysis of a sucrose synthase gene from tropical epiphytic orchid *Oncidium goldiana*. Physiol. Plantarum 118, 352-360); 5.6 U/mg (Römer, U., Schrader, H., Günther, N., Nettelstroth, N., Frommer, W.B., Elling, L. (2004) Expression,  
30 purification and characterization of recombinant sucrose synthase I from *Solanum tuberosum* L. for carbohydrate engineering. J. Biotechnology 107, 135-149), (3) the recombinant SS had to be purified by  
35 conventional methods of purification of proteins such as chromatography, electrophoresis, isoelectric focusing, etc., which, combined, prove expensive and do not guarantee purification of the protein in a homogeneous state and (4) most of the SS is sent to

inclusion bodies or is accumulated in the form of inactive aggregates as a result of the inability of the bacterium's machinery to fold the protein correctly (Miroux, B., Walker, J.E. (1996) "Over-production of  
5 proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels" *J. Mol. Biol.* 260, 289-298).

The present invention describes the development of a  
10 system based on the use of an appropriate strain of *E. coli* and on the use of a suitable expression vector that permits the large-scale production and fast and easy purification of different variants of recombinant  
15 SS in its active form. Some of these variants have greater affinity for ADP than those obtained from plant extracts and can be used both for the production of UDPG and ADPG from inexpensive substances such as sucrose, UDP and ADP.

20 Chromatographic techniques constitute a powerful tool for determining the sucrose content of complex samples such as plant extracts, sera, urine, fruit juice, wines, fruit and foodstuffs (D'Aoust, M-A., Yelle, S, Nguyen-Quock, B. (1999) Antisense inhibition of tomato  
25 fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *Plant Cell* 11, 2407-2418; Tang, G-Q., Sturm, A. (1999) Antisense repression of sucrose synthase in carrot affects growth rather than sucrose partitioning. *Plant Mol. Biol.* 41,  
30 465-479; Frias, J., Price, K.R., Fenwich, G.R., Hedley, C.L., Sorensen, H., Vidal-Valverde, C. (1996) *J. Chromatogr. A* 719, 213-219). Such techniques require highly specialized technical personnel and involve a large investment in equipment. Unfortunately,  
35 alternative methods based on hydrolysis of the sucrose molecule by the action of the enzyme invertase and subsequent spectrophotometric or fluorimetric determination of the molecules of glucose and/or fructose (Sweetlove, L.J., Burrell, M.M., ap Rees, T.

(1996) Starch metabolism in tubers of transgenic potato with increased ADPglucose pyrophosphorylase. *Biochem. J.* 320, 493-498; Stitt, M., Lilley, R.M., Gerhardt, R., Heldt, H.W. (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods Enzymol.* 174, 518-552; Holmes, E.W. (1997) Coupled enzymatic assay for the determination of sucrose. *Anal. Biochem.* 244, 103-109; *Methods of Analysis* (1996) Code of Practice for Evaluation of Fruit and Vegetable Juices. Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community) are subject to limitations of a technical nature such as subtraction of the measurements corresponding to the endogenous glucose and/or fructose present in the sample. The abundance of glucose and/or fructose in the sample can add background noise that hampers reliable and accurate determination of sucrose. In the vast majority of cases it is necessary to carry out exhaustive controls before issuing a reliable statement on the true sucrose content of a sample (Worrell, A.C., Bruneau, J-M., Summerfelt, K., Boersig, M., Voelker, T.A. (1991) Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. *Plant Cell* 3, 1121-1130). Kits for determination of sucrose based on the use of invertase are available from companies such as Sigma, Biopharm GmbH and Megazyme. Alternatively, an automated method of sucrose determination has been developed, based on determination of the glucose-1-phosphate released by the action of sucrose phosphorylase of bacterial origin (Vinet, B., Panzini, B., Boucher, M., Massicotte, J. (1998) Automated enzymatic assay for the determination of sucrose in serum and urine and its use as a marker of gastric damage. *Clin. Chem.* 44, 2369-2371). The present invention describes the development of a simple, reliable and inexpensive alternative method for the determination of sucrose in a sample based on the



use of SS and coupling enzymes which hydrolyse ADPG or UDPG.

5 Considerations concerning the factors governing the  
intracellular levels of ADPG have mainly revolved  
around regulation of the synthesizing enzyme, AGPase  
(Preiss, (1988) Biosynthesis of starch and its  
regulation. The Biochemistry of Plants. Vol. 14,  
Academic Press, New York, p. 182-249; Pozueta-Romero,  
10 J., Perata, P., Akazawa, T. (1999) Sucrose-starch  
conversion in heterotrophic tissues. Crit. Rev. Plant.  
Sci. 18, 489-525). In fact, a high proportion of the  
patents and scientific publications concerning the  
production of ADPG and the production of plants  
15 producing starches of industrial interest revolve  
around the use of AGPase (Stark, D.M., Timmerman, K.P.,  
Barry, G.F., Preiss, J., Kishore, G.M. (1992)  
Regulation of the amount of starch in plant tissues by  
ADPglucose pyrophosphorylase. Science 258, 287-282;  
20 Slattery, C.J., Kavakli, H., Okita, T.W. (2000)  
Engineering starch for increased quantity and quality.  
Trends Plant Sci. 5, 291-298). However, although they  
are yet to be confirmed with evidence of the  
genetic/molecular type, recent scientific studies of a  
25 biochemical type indicate that, as shown in Figs. 1B  
and 2B, SS might be involved in the direct synthesis of  
ADPG necessary for the biosynthesis of starch (Baroja-  
Fernández, E., Muñoz, F.J., Saikusa, T., Rodríguez-  
López, M., Akazawa, T., Pozueta-Romero, J. (2003)  
30 Sucrose synthase catalyzes the de novo production of  
ADPglucose linked to starch biosynthesis in  
heterotrophic tissues of plants. Plant Cell Physiol.  
44, 500-509). This hypothesis is especially  
controversial, bearing in mind that (a) SS has never  
35 been linked to starch production in leaves, (b)  
presence of an ADPG translocator is required in the  
membranes of the plastids, connecting the cytosolic  
pool of the ADPG produced by SS to the starch synthase  
present inside the plastid and (c) the involvement of

SS as an ADPG producing source is in direct conflict with many tests of the biochemical/genetic/molecular type which appear to show that AGPase is the only source of ADPG (Okita, T.W. (1992) Is there an alternative pathway for starch synthesis? Plant Physiol. 100, 560-56; Müller-Röber, B., Sonnewald, U. Willmitzer, L. (1992) Inhibition of the ADPglucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11, 1229-1238; Stark, D.M., Timmerman, K.P., Barry, G.F., Preiss, J., Kishore, G.M. (1992) Regulation of the amount of starch in plant tissues by ADPglucose pyrophosphorylase. Science 258, 287-282; Neuhaus, E.H., Häusler, R.E., Sonnewald, U. (2005) No time to shift the paradigm on the metabolic pathway to transitory starch in leaves. Trends Plant Sci. at press). Perhaps for all these reasons, to date plants have never been designed that overexpress SS for the production of high levels of starch. However, the present invention describes, for the first time, the production of transgenic plants that overexpress SS for increasing their production of ADPG and starch. Conversely, we show that plants that are deficient in starch as a result of absence of AGPase possess normal ADPG levels. This all shows that, as shown in Figs. 1B and 2B, SS is involved in the direct synthesis of the ADPG required for the biosynthesis of starch and is responsible for the synthesis of most of the ADPG accumulated in the plant cell.

Although based on the approach presented in Fig. 1A, according to which SS is involved in the synthesis of UDPG (but not ADPG) in storage tissues, various works have described the production of plants with reduced content of starch as a consequence of decreased activity of SS (Chourey, P.S., Nelson, O.E. (1976) The enzymatic deficiency conditioned by the shrunken-1 mutations in maize. Biochem. Genet. 14, 1041-1055;

Zrenner, R., Salanoubat, M., Willmitzer, L., Sonnewald, U. (1995) Evidence for the crucial role of sucrose synthase for sink strength using transgenic potato plants. *Plant J.* 7, 97-107; Tang, G-Q., Sturm, A. 5 (1999) Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. *Plant Mol. Biol.* 41, 465-479). In this sense, there is no experimental evidence that the overexpression of SS could be used for the production 10 of plants with high starch content as a result of the increase in levels of ADPG in accordance with the metabolic schemes shown in Figs. 1B and 2B. However, based on the ability of SS to produce the precursor molecule of the biosynthesis of cell wall 15 polysaccharides (UDPG), works have been published and patented which describe the production of cotton plants with high fibre content or cereals with high content of celluloses as a result of overexpression of SS (Timothy, H.J., Xiamomu, N., Kanwarpal, S. 20 "Manipulation of sucrose synthase genes to improve stalk and grain quality" Patent WO02067662; Robert, F., Danny, L., Yong-Ling, R. "Modification of sucrose synthase gene expression in plant tissue and uses therefor" Patent WO0245485; Christopher, B., William, 25 B., Robert, H. "Bacterial sucrose synthase compositions and methods of use" Patent WO9803637).

The invention relates firstly to the development and optimization of a method of production of large amounts 30 of recombinant SS that is soluble, can be purified easily and has high specific activity, based on the use of a suitable strain of *E. coli* and on the use of an expression vector that makes it possible to obtain SS with a histidine tail. The invention further relates to 35 the procedure followed for making kits for determination of sucrose based on the use of the enzyme product with SS activity coupled to enzymes that metabolize ADPG or UDPG. It further relates to optimization of the production of sugar nucleotides

such as ADPG or UDPG starting from variants of SS specially designed for this purpose. Finally, details are given of the design of transgenic plants with high content of sucrose, ADPG and starch and a high amylose/amylopectin ratio following overexpression of SS.

#### DETAILED DESCRIPTION OF THE INVENTION

##### 10 **Amplification of a cDNA that encodes an SS**

Knowing the nucleotide sequence of wild-type sucrose synthase SS4 (Fu, H., Park, W.D. (1995) Sink- and vascular-associated sucrose synthase functions are encoded by different gene classes in potato. Plant Cell 7, 1369-1385), two specific primers were created corresponding to the 5' and 3' ends of the gene. Using these primers, a 2418 base pair DNA fragment, designated SSX, from a potato-leaf cDNA library, was amplified by conventional PCR techniques. This PCR fragment was inserted in the pSK Bluescript plasmid (Stratagene), giving rise to the pSS construction (Fig. 3A), which was amplified in the host bacterium XL1 Blue.

##### 25 **Production of active recombinant SS from a special strain of *E. coli***

pSS was digested with the NcoI and NotI restriction enzymes. The fragment released (which contains the cDNA encoding SS, SSX) was cloned on the same restriction sites of the pET-28a(+) expression plasmid (Novagen) (Fig. 3B) which possesses a nucleotide sequence in the polylinker region that encodes a histidine-rich sequence, which becomes fused with the recombinant protein. The resulting plasmid (designated pET-SS, Fig. 3C) was inserted by electroporation in various strains of *E. coli*. The *E. coli* strain BLR(DE3) (Novagen) transformed with pET-SS was deposited in the Spanish

Type Culture Collection on 29 October 2003, located in the Research Building of Valencia University, Burjassot Campus, Burjassot 46100 (Valencia, Spain) with the deposition number CECT:5850. The bacteria were incubated at 20°C in LB medium. Overexpression of SSX was effected by addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) in 100 ml of cell culture grown at 20°C. After six hours of induced culture, the bacteria were collected and resuspended in 4 ml of binding buffer (Novagen, His-bind purification kits), then sonicated and centrifuged at 40,000 g for 20 minutes. The supernatant, which contains the recombinant SS with an amino acid sequence rich in histidine residues at the N-terminal end, was passed through an affinity column of the His-bind protein purification kit from Novagen. Following the instructions with the kit, SS was eluted with 6 ml of the recommended elution buffer, which contained 200 mM of imidazole instead of 1 mol. After elution, the protein was quickly submitted to dialysis to remove any trace of imidazole, which inactivates SS irreversibly.

**Production of an isoform of SS optimized for production of ADPG**

Using suitable primers, with pSS as template, the mutated variant SS5 was designed, giving rise to the construction pSS5. This was done using the QuikChange Site-Directed Mutagenesis kit (Stratagene). pSS5 was digested with NcoI and NotI. The fragment released (which contains SS5) was cloned on the same restriction sites of the pET-28a(+) expression plasmid giving rise to pET-SS5, which was inserted by electroporation in *E. coli* BLR(DE3). The *E. coli* strain XL1 Blue transformed with pSS5 was deposited in the Spanish Type Culture Collection on 29 October 2003, located in the Research Building of Valencia University, Burjassot Campus, Burjassot 46100 (Valencia, Spain) with the deposition number CECT:5849.

### **Production of transgenic plants that overexpress SS4**

In the present invention SS was overexpressed (a)  
5 constitutively, (b) specifically in leaves and (c)  
specifically in storage organs such as tubers.

For the production of plants that overexpress SS  
constitutively, constructions were created that were  
10 controlled by the action of the 35S constitutive  
promoter of the tobacco mosaic virus. Successive  
insertion in pSS of the 35S promoter and NOS terminator  
in the 5' and 3' regions of SSX gave rise to the  
production of the plasmid p35S-SS-NOS, the restriction  
15 map of which is shown in Fig. 4B.

So as to be able to transfer this construction to the  
genome of the plants via *Agrobacterium tumefaciens*, it  
must first be cloned in a binary plasmid. For this,  
20 p35S-SS-NOS was digested successively with the enzymes  
NotI, T4 DNA polymerase and HindIII and was cloned  
within the binary plasmid pBIN20 (Fig. 4A) (Hennegan,  
K.P., Danna, K.J. (1998) pBIN20: An improved binary  
vector for *Agrobacterium*-mediated transformation. Plant  
25 Mol. Biol. Rep. 16, 129-131) which had previously been  
digested successively with the enzymes EcoRI, T4 DNA  
polymerase and HindIII. The plasmid thus obtained was  
designated pBIN35S-SS-NOS (Fig. 4C).

30 To overexpress SS specifically in illuminated leaves,  
PCR was used for amplifying the promoter region  
(designated RBCS) of the gene that encodes the small  
subunit of RUBISCO (ribulose-1,5-bisphosphate  
carboxylase/oxygenase) of tobacco (Barnes, S.A.,  
35 Knight, J.S., Gray, J.C. (1994) Alteration of the  
amount of the chloroplast phosphate translocator in  
transgenic tobacco affects the distribution of  
assimilate between starch and sugar. Plant Physiol.  
106, 1123-1129). This nucleotide sequence (which

confers specific expression in photosynthetically active cells) was inserted in the pGEMT-easy vector (Promega), giving rise to pGEMT-RBCSprom (Fig. 5A). This construction was digested with HindIII and NcoI and the fragment released was cloned in the corresponding restriction sites of p35S-SS-NOS, giving rise to pRBCS-SS-NOS (Fig. 5B). This construction was digested successively with HindIII, T4 DNA polymerase and NotI. The fragment released was cloned in pBIN20 digested successively with HindIII, T4 DNA polymerase and EcoRI. The resulting construction was designated pBINRBCS-SS-NOS (Fig. 5C).

After being amplified in *E. coli* (XL1 Blue), both pBIN35S-SS-NOS and pBINRBCS-SS-NOS were inserted in *A. tumefaciens* C58:GV2260 (Debleare, R., Rytiebier, B., de Greve, H., Debroeck, F., Schell, J., van Montagu, M., Leemans, J. (1985) "Efficient octopine Ti plasmid-derived vectors of *Agrobacterium* mediated gene transfer to plants" Nucl. Acids Res. 13, 4777-4788), which was used for transforming species such as tomato (*Lycopersicon sculentum*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*) and rice by conventional techniques (Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T. (1985) "A simple and general method for transferring genes into plants" Science 277, 1229-1231; Pozueta-Romero, J., Houlné, G., Schantz, R., Chamarro, J. (2001) "Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation" Plant Cell Tiss. Org. Cult. 67, 173-180; Hiei, Y., Ohta, S., Komari, T., Kumashiro, T. (1994) "Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6, 271-282). The strain of *A. tumefaciens* C58:GV2260 transformed with pBIN35S-SS-NOS was deposited in the Spanish Type Culture Collection on 29 October 2003, located in the Research Building of Valencia University, Burjassot Campus, Burjassot 46100

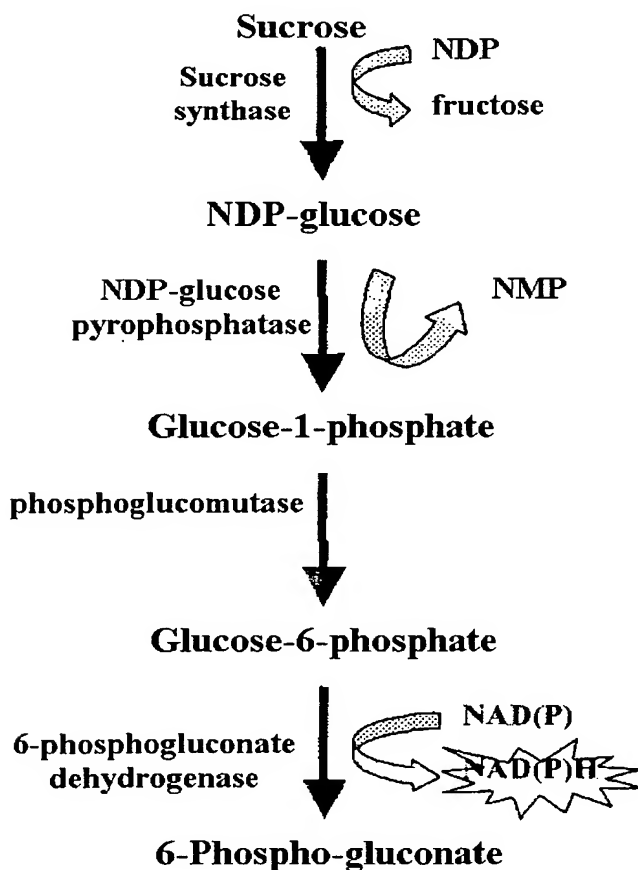
(Valencia, Spain), with the deposition number CECT:5851.

### Preparation of assay kits for determination of sucrose

5

One of the kits designed for the determination of sucrose, shown in the following Scheme I of enzymatic reactions involved in the kit for spectrophotometric/fluorimetric determination of sucrose based on the conversion of sucrose to a sugar nucleotide and then conversion of this to glucose-1-phosphate, glucose-6-phosphate and NAD(P)H.

10



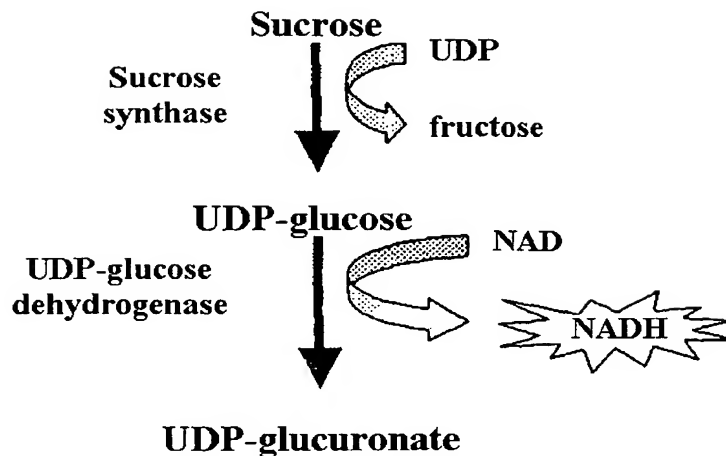
The kit is based on the action of SS on the sucrose molecule in the presence of a nucleotide diphosphate (e.g. UDP or ADP), releasing equimolar amounts of fructose and the corresponding sugar nucleotide. If the

15



sugar nucleotide resulting from the reaction is UDPG, this is submitted to the action of hydrolytic enzymes of UDPG such as UDPG pyrophosphatase of the Nudix type (EC 3.6.1.45) (Yagi, T., Baroja-Fernández, E., Yamamoto, R., Muñoz, F.J., Akazawa, T., Pozueta-Romero, J. (2003) Cloning, expression and characterization of a mammalian Nudix hydrolase-like enzyme that cleaves the pyrophosphate bond of UDP-glucose. *Biochem. J.* 370, 409-415) or UDPG hydrolase (Burns, D.M., Beacham, I.R. (1986) Nucleotide sequence and transcriptional analysis of the *E. coli* *ushA* gene, encoding periplasmic UDP-sugar hydrolase (5'-nucleotidase): regulation of the *ushA* gene, and the signal sequence of its encoded protein product. *Nucl. Acids Res.* 14, 4325-4342). The GlP released by the action of these hydrolytic enzymes is transformed by the action of phosphoglucomutase (PGM), yielding glucose-6-phosphate (G6P), which in its turn can be made to undergo a coupling reaction with NAD(P)<sup>+</sup> by the action of the enzyme G6P dehydrogenase (G6PDH), producing 6-phosphogluconate and NAD(P)H, which can easily be determined by fluorimetry and by spectrophotometry at 340 nm. In its turn, the NAD(P)H released can be coupled to the action of FMN-oxidoreductase/luciferase, yielding light, which is quantified spectrophotometrically.

Alternatively, as shown in scheme II, the UDPG produced can be coupled with UDPG dehydrogenase (EC 1.1.1.22) which, in the presence of NAD, gives rise to equimolar amounts of UDP-glucuronate and NADH, which can be determined by fluorimetry or by spectrophotometry at 340 nm. In its turn, the NADH released can be coupled to the action of FMN-oxidoreductase/luciferase, yielding light, which is quantified spectrophotometrically.



**Scheme II**

If the product of the reaction catalysed by the SS is ADPG, this is submitted to the action of hydrolytic enzymes of ADPG such as bacterial ADPG pyrophosphatase (EC 3.6.1.21) (Moreno-Bruna, B., Baroja-Fernández, E., Muñoz, F.J., Bastarrica-Berasategui, A., Zandueta-Criado, A., Rodríguez-López, M., Lasa, I., Akazawa, T., Pozueta-Romero, J. (2001) Adenosine diphosphate sugar pyrophosphatase prevents glycogen biosynthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 98, 8128-8132). The G1P released is transformed by the action of phosphoglucomutase, yielding glucose-6-phosphate (G6P), which can in turn be made to undergo a coupling reaction with NAD(P)<sup>+</sup> by the action of the enzyme G6P dehydrogenase, producing 6-phosphogluconate and NAD(P)H, which can easily be determined by fluorimetry or spectrophotometry at 340 nm.

In any case, the schemes of enzymatic reactions coupled to the production of a sugar nucleotide mediated by SS are perfectly suitable for application to amperometric detection.

#### EXAMPLES OF CARRYING OUT THE INVENTION

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Examples are described below, which show in detail the procedure for cloning a cDNA that encodes an isoform of

SS of potato in a suitable expression vector and in a strain of *E. coli* optimized for the production and accumulation of the enzyme in its active form. Other examples describe the use of the recombinant SS for making assay kits for the determination of sucrose in plant samples, serum, urine, fruit juices, sweetened fruit drinks, refreshing drinks, etc. Another example describes the use of variants of SS optimized for the large-scale production of sugar nucleotides such as UDPG and ADPG. Finally, another example describes the production of plants with high content of sucrose, ADPG and starch and a high amylose/amylopectin ratio as a result of the high ADPG-producing activity in plants that overexpress SS.

15

**Example 1: Expression, in *Escherichia coli* BLR (DE3), of a recombinant SS with a histidine tail, which can be purified easily and has high specific activity**

20 Knowing the nucleotide sequence of the SS4 gene that encodes an isoform of SS of potato, it was possible to create two specific primers whose sequences are, in the 5' - 3' direction, SEQ ID NO: 1 and SEQ ID NO: 2. Using these primers, a DNA fragment, designated as SSX, was amplified by conventional methods of PCR, from a potato tuber cDNA library, and this was inserted in a pSK Bluescript plasmid (Stratagene), which was amplified in the host bacterium XL1 Blue. The nucleotide sequence of SSX is SEQ ID NO: 3, which is slightly different from SS4 (GenBank accession number U24087). The amino acid sequence deducted from SEQ ID NO: 3 is slightly different from SS4 and is therefore designated SSX. The amino acid sequence deducted after expression of SEQ ID NO: 3 in the pET-28a(+) plasmid is SEQ ID NO: 4, which includes a histidine-rich sequence of 38 amino acids fused with the amino-terminal end of the amino acid sequence deducted from SEQ ID NO: 3.

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Production of SSX in BL21(DE3) bacteria transformed with pET-SS was induced on adding 1 mM IPTG. After six additional hours of culture at 37°C, it was observed that the bacteria transformed with pET-SS accumulated a protein in aggregated form, the size of which corresponds to SS. However, these bacteria did not have SS activity. This failure in the expression of an active form of SS can be attributed to the problems that *E. coli* has in the correct folding of certain eukaryotic proteins of high molecular weight (Miroux, B., Walker, J.E. (1996) "Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels" J. Mol. Biol. 260, 289-298). With the aim of overcoming this problem, the capacity for production of active SS in other bacterial strains and at a temperature of 20°C was investigated. In all of them, production of SSX was induced on adding 1 mM of IPTG. After 6 hours of additional incubation, the bacteria were sonicated and centrifuged. The resulting supernatant was analysed for SS activity. In these conditions, as shown in Fig. 6, the BLR(DE3) strain proved to be the most efficient from the standpoint of production of soluble, active SS. The *E. coli* strain BLR(DE3) (Novagen) transformed with pET-SS was deposited in the Spanish Type Culture Collection on 29 October 2003, with the deposition number CECT:5850. The contribution of recombinant SSX in the total protein pool of CECT:5850 is approximately 20%, compared to the very low productivity of recombinant SS (30 micrograms per gram of bacteria) described in the literature (Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F., Hayashi, T. (1997) "Expression and characterization of sucrose synthase from mung bean seedlings in *Escherichia coli*" Biosci. Biotech. Biochem. 61, 1500-1503; Li, C.R., Zhang, X.B., Hew, C.S. (2003) "Cloning, characterization and expression analysis of a sucrose synthase gene from tropical epiphytic orchid *Oncidium goldiana*. Physiol. Plantarum

118, 352-360). The supernatant was passed through the His-Bind affinity column (Novagen), in which the recombinant protein possessing a histidine tail is retained specifically. After eluting and dialysing the purified SS, it was incubated with 50 mM HEPES, pH 7.0 / 1 mM EDTA / 20% polyethylene glycol / 1 mM MgCl<sub>2</sub> / 15 mM KCl / 2 mM UDP. The specific activity, determined in terms of production of UDPG, was 80 units/mg of protein, much higher than the activity of 0.05-5 units/mg of recombinant SS described in the literature (Nakai, T., Tonouchi, N., Tsuchida, T., Mori, H., Sakai, F., Hayashi, T. (1997) "Expression and characterization of sucrose synthase from mung bean seedlings in *Escherichia coli*" Biosci. Biotech. Biochem. 61, 1500-1503; Li, C.R., Zhang, X.B., Hew, C.S. (2003) "Cloning, characterization and expression analysis of a sucrose synthase gene from tropical epiphytic orchid *Oncidium goldiana*. Physiol. Plantarum 118, 352-360); Römer, U., Schrader, H., Günther, N., Nettelstroth, N., Frommer, W.B., Elling, L. (2004) Expression, purification and characterization of recombinant sucrose synthase I from *Solanum tuberosum* L. for carbohydrate engineering. J. Biotechnology 107, 135-149) and greater than 3 units/mg corresponding to the SS purified from plant extracts (Pressey R (1969) Potato sucrose synthase: purification, properties, and changes in activity associated with maturation. Plant Physiol. 44, 759-764. The unit is defined as the amount of enzyme that catalyses the production of one micromol of UDPG per minute. The affinity for UDP in the presence of 500 mM sucrose was  $K_m(\text{UDP}) = 0.25 \text{ mM}$ , whereas the  $K_m$  for sucrose was 30 mM in the presence of 1 mM UDP. This affinity for sucrose in the presence of UDP is significantly higher than that exhibited by the recombinant SS obtained in yeasts ( $K_m = 95 \text{ mM}$ , Römer, U., Schrader, H., Günther, N., Nettelstroth, N., Frommer, W.B., Elling, L. (2004) Expression, purification and characterization of recombinant sucrose synthase I from *Solanum tuberosum* L. for

carbohydrate engineering. J. Biotechnology 107, 135-149).

**Example 2: Large-scale production of UDPG and ADPG  
based on the use of recombinant SS from E. coli**

Three grams of UDPG of high purity was produced efficiently and economically after incubation for 12 hours at 37°C of 100 millilitres of a solution containing 1 M sucrose, 50 mM HEPES, pH 7.0 / 1 mM EDTA / 20% polyethylene glycol / 1 mM MgCl<sub>2</sub> / 15 mM KCl / 100 mM UDP and 30 units of recombinant SS from potato obtained after expression of pET-SS in BLR(DE3) and subsequent purification. Reaction came to an end after heating the solution at 100°C for 90 seconds and then centrifugation at 10,000 g for 10 minutes. The supernatant was applied to a preparative-scale HPLC chromatograph (Waters Associates) and the UDPG was purified as described in the literature (Rodríguez-López, M., Baroja-Fernández, E., Zanduetta-Criado, A., Pozueta-Romero, J. (2000) Adenosine diphosphate glucose pyrophosphatase: a plastidial phosphodiesterase that prevents starch biosynthesis. Proc. Natl. Acad. Sci. USA 97, 8705-8710).

Production of ADPG required the generation of a mutated form of SS with an affinity for ADP much greater than that described for the SS extracted from plant tissues (Pressey R (1969) Potato sucrose synthase: purification, properties, and changes in activity associated with maturation. Plant Physiol. 44, 759-764; Nguyen-Quock, B., Krivitzky, M., Huber, S.C., Lecharny, A. (1990) Sucrose synthase in developing maize leaves. Plant Physiol. 94, 516-523; Morell, M., Copeland, L. (1985) Sucrose synthase of soybean nodules. Plant Physiol. 78, 149-154).

This isoform, designated SS5, was obtained by point mutagenesis of SSX using the QuikChange Site-Directed

Mutagenesis kit (Stratagene) and successive use of the following pairs of primers whose sequences are [SEQ ID NO: 5, SEQ ID NO: 6], [SEQ ID NO: 7, SEQ ID NO: 8] and [SEQ ID NO: 9, SEQ ID NO: 10]. The nucleotide sequence  
5 obtained, designated SS5, is SEQ ID NO: 11. The changes in the amino acid sequence of SS5 (Susy 5) relative to SS4 -Susy 4- (present in databases) are shown shaded in Table I. The amino acid sequence deducted after  
10 expression of SEQ ID NO: 11 in the pET-28a(+) plasmid is SEQ ID NO: 12, which includes a histidine-rich sequence of 38 amino acids fused with the amino-terminal end of the amino acid sequence deducted from SEQ ID NO: 11.

15 Table I includes said histidine-rich sequence of 38 amino acids fused to the amino-terminal portion of SS5.

1	M	C	A	S	H	H	H	H	N	S	S	G	L	V	P	R	G	S	H	M	A	S	M	T	G	G	O	M	Susy 4		
	-	-	-	-	-	M	A	E	R	V	L	T	R	V	H	S	L	R	E	R	V	D	A	T	L	A	A	H	R	Susy 5	
2	G	R	G	S	E	F	M	A	E	R	V	L	T	R	V	H	S	L	R	E	R	V	D	A	T	L	A	A	H	R	Susy 4
31																													Susy 5		
25	N	E	I	L	L	F	L	S	R	I	E	S	H	G	K	G	I	L	K	P	H	E	L	L	A	E	F	D	A	I	Susy 4
61	N	E	I	L	L	F	L	S	R	I	E	S	H	G	K	G	I	L	K	P	H	E	L	L	A	E	F	D	A	I	Susy 5
55	R	Q	D	D	K	N	K	L	N	E	H	A	F	E	E	L	L	K	S	T	Q	E	A	I	V	L	P	P	W	V	Susy 4
91	R	Q	D	D	K	N	K	L	N	E	H	A	F	E	E	L	L	K	S	T	Q	E	A	I	V	L	P	P	W	V	Susy 5
85	A	L	A	I	R	L	R	P	G	V	W	E	Y	I	R	V	N	V	N	A	L	V	V	E	E	L	S	V	P	E	Susy 4
121	A	L	A	I	R	L	R	P	G	V	W	E	Y	I	R	V	N	V	N	A	L	V	V	E	E	L	S	V	P	E	Susy 5
115	Y	L	Q	F	K	E	E	L	V	D	G	A	S	N	G	N	F	V	L	E	L	D	F	E	F	F	T	A	S	F	Susy 4
151	Y	L	Q	F	K	E	E	L	V	D	G	A	S	N	G	N	F	V	L	E	L	D	F	E	F	F	T	A	S	F	Susy 5
145	P	K	P	T	L	T	K	S	I	G	N	G	V	E	F	L	N	R	H	L	S	A	K	M	F	H	D	K	E	S	Susy 4
181	P	K	P	T	L	T	K	S	I	G	N	G	V	E	F	L	N	R	H	L	S	A	K	M	F	H	D	K	E	S	Susy 5
175	M	T	P	L	L	E	F	L	R	A	H	H	Y	K	G	K	T	M	M	L	N	D	R	I	Q	N	S	N	T	L	Susy 4
211	M	T	P	L	L	E	F	L	R	A	H	H	Y	K	G	K	T	M	M	L	N	D	R	I	Q	N	S	N	T	L	Susy 5
205	Q	N	V	L	R	K	A	E	E	Y	L	I	M	L	P	P	E	T	P	Y	F	E	F	E	H	K	F	Q	E	I	Susy 4
241	Q	N	V	L	R	K	A	E	E	Y	L	I	M	L	S	P	D	T	P	Y	F	E	F	E	H	K	F	Q	E	I	Susy 5
235	G	L	E	K	G	W	G	D	T	A	E	R	V	L	E	M	V	C	M	L	L	D	L	L	E	A	P	D	S	C	Susy 4
271	G	L	E	K	G	W	G	D	T	A	E	R	V	L	E	M	V	C	M	L	L	D	L	L	E	A	P	D	S	C	Susy 5
265	T	L	E	K	F	L	G	R	I	P	M	V	F	N	V	V	I	L	S	P	H	G	Y	F	A	Q	E	N	V	L	Susy 4
301	T	L	E	K	F	L	G	R	I	P	M	V	F	N	V	V	I	L	S	P	H	G	Y	F	A	Q	E	N	V	L	Susy 5
295	G	Y	P	D	T	G	G	Q	V	V	Y	I	L	D	Q	V	P	A	L	E	R	E	M	L	K	R	I	K	E	Q	Susy 4
331	G	Y	P	D	T	G	G	Q	V	V	Y	I	L	D	Q	V	P	A	L	E	R	E	M	L	K	R	I	K	E	Q	Susy 5
325	G	L	D	I	I	P	R	I	L	I	V	T	R	L	L	P	D	A	V	G	T	T	C	G	Q	R	I	E	K	V	Susy 4
361	G	L	D	I	I	P	R	I	L	I	V	T	R	L	L	P	D	A	V	G	T	T	C	G	Q	R	I	E	K	V	Susy 5
355	Y	G	A	E	H	S	H	I	L	R	V	P	F	R	T	E	K	G	I	V	R	K	W	I	S	R	F	E	V	W	Susy 4
391	Y	G	A	E	H	S	H	I	L	R	V	P	F	R	T	E	K	G	I	V	R	K	W	I	S	R	F	E	V	W	Susy 5
385	P	Y	M	E	T	F	I	E	D	V	A	K	E	I	S	A	E	L	Q	A	K	P	D	L	I	I	G	N	Y	S	Susy 4
421	P	Y	M	E	T	F	I	E	D	V	A	K	E	I	S	A	E	L	Q	A	K	P	D	L	I	I	G	N	Y	S	Susy 5
415	E	G	N	L	A	A	S	L	L	A	H	K	L	G	V	T	Q	C	T	I	A	H	A	L	E	K	T	K	Y	P	Susy 4
451	E	G	N	L	A	A	S	L	L	A	H	K	L	G	V	T	Q	C	T	I	A	H	A	L	E	K	T	K	Y	P	Susy 5
445	D	S	D	I	Y	W	K	K	F	D	E	K	Y	H	F	S	S	Q	F	T	A	D	L	I	A	M	N	H	T	D	Susy 4
481	D	S	D	I	Y	W	K	K	F	D	E	K	Y	H	F	S	S	Q	F	T	A	D	L	I	A	M	N	H	T	D	Susy 5
475	F	I	I	T	S	T	F	Q	E	I	A	G	S	K	D	T	V	G	Q	Y	E	S	H	M	A	F	T	M	P	G	Susy 4
511	F	I	I	T	S	T	F	Q	E	I	A	G	S	K	D	T	V	G	Q	Y	E	S	H	M	A	F	T	M	P	G	Susy 5
505	L	Y	R	V	V	H	G	I	N	V	F	D	P	K	F	N	I	V	S	P	G	A	D	I	N	L	Y	F	S	Y	Susy 4
541	L	Y	R	V	V	H	G	I	N	V	F	D	P	K	F	N	I	V	S	P	G	A	D	I	N	L	Y	F	S	Y	Susy 5
535	S	E	T	E	K	R	L	T	A	F	H	P	E	I	D	E	L	L	Y	S	D	V	E	N	D	E	H	L	C	V	Susy 4
571	S	E	T	E	K	R	L	T	A	F	H	P	E	I	D	E	L	L	Y	S	D	V	E	N	D	E	H	L	C	V	Susy 5
565	L	K	D	R	T	K	P	I	L	F	T	M	A	R	L	D	R	V	K	N	L	T	G	L	V	E	W	Y	A	K	Susy 4
601	L	K	D	R	T	K	P	I	L	F	T	M	A	R	L	D	R	V	K	N	L	T	G	L	V	E	W	Y	A	K	Susy 5
595	N	P	R	L	R	G	L	V	N	L	V	V	V	G	G	D	R	R	K	E	S	K	D	L	E	E	Q	A	E	M	Susy 4
631	N	P	R	L	R	G	L	V	N	L	V	V	V	G	G	D	R	R	K	E	S	K	D	L	E	E	Q	A	E	M	Susy 5
625	K	K	M	Y	E	L	I	E	T	H	N	L	N	G	Q	F	R	W	I	S	S	Q	M	N	R	V	R	N	G	E	Susy 4
661	K	K	M	Y	E	L	I	E	T	H	N	L	N	G	Q	F	R	W	I	S	S	Q	M	N	R	V	R	N	G	E	Susy 5
655	L	Y	R	Y	I	A	D	T	K	G	A	F	V	Q	P	A	F	Y	E	A	F	G	L	T	V	V	E	A	M	T	Susy 4
691	L	Y	R	Y	I	A	D	T	K	G	A	F	V	Q	P	A	F	Y	E	A	F	G	L	T	V	V	E	A	M	T	Susy 5
685	C	G	L	P	T	F	A	T	N	H	G	G	P	A	E	I	I	V	H	G	K	S	G	F	H	I	D	P	Y	H	Susy 4
721	C	G	L	P	T	F	A	T	N	H	G	G	P	A	E	I	I	V	H	G	K	S	G	F	H	I	D	P	Y	H	Susy 5
715	G	E	Q	A	A	D	L	L	A	D	F	F	E	K	C	K	K	D	P	S	H	W	E	T	I	S	M	G	G	L	Susy 4
751	G	E	Q	A	A	D	L	L	A	D	F	F	E	K	C	K	K	D	P	S	H	W	E	T	I	S	T	D	G	L	Susy 5
745	K	R	I	E	E	K	Y	T	W	Q	I	Y	S	E	S	L	L	T	L	A	A	V	Y	G	F	W	K	H	V	S	Susy 4
781	K	R	I	E	E	K	Y	T	W	Q	I	Y	S	E	S	L	L	T	L	A	A	V	Y	G	F	W	K	H	V	S	Susy 5
775	K	L	D	R	L	E	I	R	R	Y	L	E	M	F	Y	A	L	K	Y	R	K	M	A	E	A	V	P	L	A	A	Susy 4
811	K	L	D	R	L	E	I	R	R	Y	L	E	M	F	Y	A	L	K	Y	R	K	M	A	E	A	V	P	L	A	A	Susy 5
805	E																													Susy 4	
841	E																													Susy 5	



The recombinant SS5 obtained after expression of pET-SS5 had a  $V_{max}$  of 80 units/mg of protein and 65 units/mg of protein in the presence of UDP and ADP, respectively. The affinities for UDP and ADP in the presence of 500 mM sucrose were very similar ( $K_m = 0.2$  mM both for ADP and for UDP), whereas the  $K_m$  for sucrose was 30 mM and 100 mM in the presence of saturated concentrations of UDP and ADP, respectively. These kinetic parameters are very different from those described for the SS extracted from potato tuber and other organs of other species, according to which the  $V_{max}$  of the enzyme is 10 times higher in the presence of UDP than in the presence of ADP (Pressey R (1969) Potato sucrose synthase: purification, properties, and changes in activity associated with maturation. Plant Physiol. 44, 759-764; Morell, M., Copeland, L. (1985) Sucrose synthase of soybean nodules. Plant Physiol. 78, 149-154; Nguyen-Quock, B., Krivitzky, M., Huber, S.C., Lecharny, A. (1990) Sucrose synthase in developing maize leaves. Plant Physiol. 94, 516-523). The *E. coli* strain XL1 Blue transformed with pSS5 was deposited in the Spanish Type Culture Collection, with the deposition number CECT:5849.

Three grams of ADPG of high purity was produced efficiently and economically after incubation for 12 hours at 37°C of 100 millilitres of a solution containing 1 M sucrose, 50 mM HEPES, pH 7.0 / 1 mM EDTA / 20% polyethylene glycol / 1 mM  $MgCl_2$  / 15 mM KCl / 100 mM ADP and 30 units of recombinant SS from potato obtained after expression of pET-SS5 in BLR(DE3) and subsequent purification in a His-bind column. Reaction came to an end after heating the solution at 100°C for 90 seconds and then centrifugation at 10,000 g for 10 minutes. The supernatant was applied to a preparative-scale HPLC chromatograph (Waters Associates) for purification of the ADPG.

**Example 3: Preparation of enzymatic kits for determination of sucrose**

For determination of sucrose, the following reaction  
5 cocktails were prepared with the following components  
and final amounts/concentrations:

1. KITS BASED ON THE USE OF HYDROLYTIC ENZYMES OF SUGAR  
NUCLEOTIDES:

- 10 a. 2 units of SS (recombinant or not)
- b. 2 mM of ADP or UDP (depending on whether ADPG or  
UDPG is being produced, respectively)
- c. 2 units of ADPG pyrophosphatase or 2 units of UDPG  
pyrophosphatase (depending on whether it is to be  
15 included in the ADP or UDP reaction cocktail,  
respectively)
- d. 2 units of PGM
- e. 2 units of G6PDH
- f. 0.5 mM of NAD(P)
- 20 g. reaction buffer: 50 mM HEPES, pH 7.0 / 1 mM EDTA /  
20% polyethylene glycol / 1 mM  $\text{MgCl}_2$  / 15 mM KCl
- h. previously filtered test sample

2. KIT BASED ON THE USE OF UDPG DEHYDROGENASE

- 25 a. 2 units of SS (recombinant or not)
- b. 2 mM of UDP
- c. 2 units of UDPG dehydrogenase
- d. 0.5 mM of NAD
- e. reaction buffer: 50 mM HEPES, pH 7.0 / 1 mM EDTA /  
30 20% polyethylene glycol / 1 mM  $\text{MgCl}_2$  / 15 mM KCl
- f. previously filtered test sample

Determination of the amount of sucrose present in the  
test sample is based on fluorimetric determination or  
35 spectrophotometric determination (at 340 nm) of the  
NAD(P)H produced according to the coupled reactions  
shown in schemes I and II.

For determining the sucrose content of barley seeds with different degrees of development (Fig. 7), the reactions took place in 300-microlitre wells of an ELISA plate for 3 minutes at 37°C. The volume of the test sample was 20 microlitres, and the volume of the cocktail resulting from combination of reagents a-g (kit #1) and a-e (kit #2) was 280 microlitres. The blanks contained all the components of the cocktail except SS. Measurement was carried out with a MultiSkan spectrophotometer. The values obtained, both with the kit of type "1" and with the kit of type "2" were found to be comparable to those determined using chromatographic techniques described in the introduction (Baroja-Fernández, E., Muñoz, F.J., Saikusa, T., Rodríguez-López, M., Akazawa, T., Pozueta-Romero, J. (2003) Sucrose synthase catalyzes the de novo production of ADPGlucose linked to starch biosynthesis in heterotrophic tissues of plants. Plant Cell Physiol. 44, 500-509).

**Example 4: Production of transgenic plants that overexpress SS**

Figs. 8-10 present the results obtained in leaves of potato plants that overexpress SS both constitutively (35S-SS-NOS), and specifically (RBCS-SS-NOS).

As shown in Fig. 8, the SS activity in the leaves of any of these plants is 2-10 times higher than in the same organ of a wild-type plant (WT). These leaves had the following characteristics:

1. Clear correlation between the ADPG-producing SS activity (Fig. 8) and levels of starch (Fig. 9) and ADPG (Fig. 10). This characteristic was observed not only in leaves, but also in storage tissues such as tubers and seeds (see below).
2. High starch content (Fig. 9) relative to leaves of wild-type plants. For example, the starch content of

- a leaf of a "wild-type" potato plant grown in a photoperiod of 8 hours light/16 hours darkness and at 20°C is 5 micromol/gram of fresh weight, whereas a leaf of a transgenic plant that overexpresses SS is 8 micromol/gram fresh weight. The differences between wild-type and transgenic plants are accentuated when the photoperiod is long, so that the leaves of a plant that overexpresses SS contains 4 times more starch than those of a wild-type plant.
3. High ADPG content relative to the same tissue or organ of the untransformed plant (Fig. 10). The average content in a leaf of a wild-type potato plant grown in a photoperiod of 8 hours light/16 hours darkness and at 20°C is 0.35 nanomol/gram of fresh weight, whereas the leaves of the plants that overexpress SS can have a content of 2.5 nanomol/gram of fresh weight.
4. Both ADPG and starch exhibit transitory accumulation over the photoperiod (Fig. 11). The rate of accumulation of both substances maintains a positive correlation with the SS activity, indicating that, contrary to what is suggested by the "classical" model of starch biosynthesis (Fig. 2A) and confirming the hypothesis of the "alternative" model shown in Fig. 2B, SS plays a fundamental role in the production of ADPG and in the link between sucrose metabolism and starch metabolism.
5. Normal levels of soluble sugars such as glucose and fructose. However, the levels of glucose-6-P and sucrose in transgenic leaves are higher than those observed in the wild-type potato leaves (Table 2).

**Table 2:** Levels of metabolites (expressed in nmol/g fresh weight) in leaves of control plants (WT) and 35S-SuSy-NOS source leaves. Values significantly different from those observed in WT are shown in bold.

	Control	35S-SS-NOS					
	WT	6	5	12	3	4	7
<u>Glucose</u>	848 ± 31	922 ± 29	860 ± 30	933 ± 29	881 ± 56	895 ± 32	871 ± 60
<u>Fructose</u>	996 ± 43	1,035 ± 67	1,094 ± 17	1,022 ± 10	1067 ± 58	1078 ± 63	817 ± 41
<u>Sucrose</u>	1,012 ± 27	1,529 ± 48	1,402 ± 68	1,642 ± 58	1,307 ± 35	1,317 ± 35	1,391 ± 70
<u>Glucose-6-P</u>	244 ± 28	309 ± 15	280 ± 25	271 ± 27	355 ± 23	298 ± 12	315 ± 9.8
<u>Glucose-1-P</u>	<u>22.7 ± 1.9</u>	<u>15.5 ± 2.1</u>	<u>10.3 ± 1.1</u>	<u>9.9 ± 1.2</u>	<u>9.5 ± 1.5</u>	<u>15.2 ± 1.9</u>	<u>11.4 ± 1.8</u>

6. The external morphology of the plants that overexpress SS is not aberrant, when compared with that of the untransformed plants.

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Figs. 12-14 show the results obtained in potato tubers that overexpress SS constitutively (35S-SS-NOS). These results are essentially identical to those observed in tubers that overexpress SS under the control of a specific tuber promoter (promoter of the patatina gene).

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As shown in Fig. 12, the SS activity in the tubers of any of these plants is ??? times greater than in the same organ of a wild-type plant. These tubers had the following characteristics:

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1. Clear correlation between the ADPG-producing SS activity (Fig. 12) and levels of starch (Fig. 13) and ADPG (Fig. 14).
2. High starch content (Fig. 13) relative to tubers of untransformed plants. For example, the starch content in the tuber of the "wild-type" plant is approximately 300 micromol/gram of fresh weight (equivalent to 54 mg of starch/gram of fresh

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weight), whereas in a tuber that overexpresses SS it is 450-600 micromol/gram fresh weight.

3. High ADPG content relative to tubers of wild-type plants (Fig. 14). The average content in a wild-type tuber is 5 nanomol/gram of fresh weight, whereas the tubers that overexpress SS can have a content of 7-9 nanomol/gram of fresh weight.

The results obtained in rice seeds, tomato and tobacco leaves, as well as tomato fruits, are qualitatively similar to those shown in Figs. 8-14. In all cases there was an increase in the content of starch and an increase in the amylose/amylopectin ratio.

The production of plants with high content of ADPG and starch following overexpression of SS is a result that is totally unexpected according to the current ideas on the biosynthesis of starch (illustrated in Figs. 1A and 2A) and perhaps explains why the design of plants that overexpress SS has not previously been adopted as a strategy for increasing starch production. The results obtained on the basis of this work suggest that SS, but not AGPase, is the fundamental source of ADPG that accumulates in plants. According to the models that are still current, AGPase is the only source of ADPG. Surprisingly, however, ADPG levels have never been investigated in AGPase-deficient plants. To explore the significance of our invention, we analysed the levels of ADPG and starch in Arabidopsis and potato plants with reduced AGPase activity for the first time. As shown in Fig. 15A, the levels of starch in AGPase-deficient TL25 Arabidopsis plants (Lin, T.P., Caspar, T., Somerville, C.R., Preiss, J. (1988) Isolation and characterization of a starchless mutant of Arabidopsis thaliana lacking ADPglucose pyrophosphorylase activity. Plant Physiol. 88, 1131-1135) are lower than those observed in the WT plants. However, the levels of ADPG are normal (Fig. 15B). In contrast, the levels of starch in AGP62 and AGP85 potato plants (Müller-Röber,

B., Sonnewald, U. Willmitzer, L. (1992) Inhibition of the ADPglucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11, 1229-1238) are reduced relative to those observed in leaves of wild-type plants (Fig. 16A). However, the levels of ADPG are completely normal (Fig. 16B). Taken together, these observations (a) show that SS, and not AGPase, is the principal source of ADPG in plants and (b) highlight the significance of our invention after demonstrating that overexpression of SS gives rise to plants with high starch content.

#### DESCRIPTION OF THE DIAGRAMS

Fig. 1: Mechanisms of starch biosynthesis in heterotrophic organs. (A) "Classical" mechanism according to which SS is involved in the production of UDPG, which is eventually converted to starch after the combined action of UDPG pyrophosphorylase (UGPase), cytosolic phosphoglucomutase (PGM), plastidial phosphoglucomutase, ADPG pyrophosphorylase (AGPase) and starch synthase. (B) "Alternative" mechanism according to which SS is involved in the direct production of ADPG in the cytosol. The ADPG is then transported to the amyloplast by the action of a translocator. Once inside the amyloplast, the starch synthase utilizes the ADPG for producing starch.

Fig. 2: Mechanisms of biosynthesis of starch in leaves. (A) "Classical" mechanism according to which the entire process of starch biosynthesis takes place inside the chloroplast. According to this view, starch metabolism and sucrose are not connected. Moreover, SS does not take part in the gluconeogenic process. (B) "Alternative" mechanism of starch biosynthesis according to which SS is involved in the direct synthesis of ADPG in the cytosol. The ADPG is then transported to the interior of the plastid where the

starch synthase utilizes it as substrate for the reaction of starch synthesis.

Fig. 3: Stages in construction of the pET-SS expression  
5 plasmid from pET-28a(+) and pSS.

Fig. 4: Stages in construction of the pBIN35S-SS-NOS expression plasmid from pBIN20 and p35S-SS-NOS.

10 Fig. 5: Stages in construction of the pRBCS-SS-NOS expression plasmid from pGEMT-RBCSprom, p35S-SS-NOS and pBIN20.

Fig. 6: Expression of pET-SS in different strains of  
15 *Escherichia coli*. (A) SS activity (in milliunits (mU) per milligram of bacterial protein) in bacterial extracts transformed with pET or with pET-SS. The reaction took place in the direction of degradation of sucrose and production of ADPG. The reaction cocktail  
20 contained 50 mM HEPES (pH 7.0), 1 mM EDTA, 20% polyethylene glycol, 1 mM MgCl<sub>2</sub>, 15 mM of KCl and 2 mM of ADP. Reaction took place for 10 minutes at 37°C. (B) SDS-PAGE of protein extracts from the various strains of *E. coli* transformed with pET and with pET-SS. The position of the recombinant SSX is indicated  
25 with an asterisk.

Fig. 7: Determination of sucrose at different stages of development of barley endosperm using the kit based on  
30 the coupled reactions of SS, ADPG (UDPG) pyrophosphatase, PGM and G6PDH. The results were identical to those obtained in parallel by (a) use of a kit based on the coupled reactions of SS and UDPG dehydrogenase and (b) use of high-performance  
35 chromatography (HPLC) with amperometric detection in a DX-500 Dionex system connected to a Carbo-Pac PA1 column.

Abscissa: Days after flowering

Ordinate: Sucrose content (μmol/gFW)



Fig. 8: SS activity in leaves of wild-type (WT) potato plants and potato plants that overexpress SSX following integration of the constructions 35S-SS-NOS (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851) or RBCS-SS-NOS in their genome. Activity is expressed in milliunits (mU) per gram of fresh weight. The unit is defined as the amount of SS required for producing one micromol of ADPG per minute.

Fig. 9: Content of starch in leaves of wild-type (WT) potato plants and potato plants that overexpress SSX following integration of the constructions 35S-SS-NOS (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851) or RBCS-SS-NOS in their genome.

Fig. 10: Content of ADPG in leaves of wild-type (WT) potato plants and potato plants that overexpress SSX following integration of the constructions 35S-SS-NOS (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851) or RBCS-SS-NOS in their genome.

Fig. 11: Transitory accumulation of (A) starch and (B) ADPG during a photoperiod of 8 hours of light and 16 hours of darkness in leaves of WT plants (●), 35S-SS-NOS (■) and RBCS-SS-NOS (▲).

Fig. 12: SS activity (referred to fresh weight, FW) in tubers of wild-type potato plants (WT), regeneration controls (RG) and potato plants that overexpress SSX (lines 4, 5, 6 and 12) after integration of the construction 35S-SS-NOS in their genome (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851). The activity is expressed in milliunits (mU) per gram of fresh weight. The unit is defined as the amount of SS required for producing one micromol of ADPG per minute.

Fig. 13: Content of starch (referred to fresh weight, FW) in tubers of wild-type potato plants (WT), regeneration controls (RG) and potato plants that overexpress SSX (lines 4, 5, 6 and 12) after  
5 integration of the construction 35S-SS-NOS in their genome (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851).

Fig. 14: Content of ADPG (referred to fresh weight, FW)  
10 in tubers of wild-type potato plants (WT) and potato plants that overexpress SSX after integration of the construction 35S-SS-NOS in their genome (by the action of the strain of *Agrobacterium tumefaciens* CECT:5851).

15 Fig. 15: Content of (A) starch and (B) ADPG in leaves of AGPase-deficient *Arabidopsis thaliana* TL25.

Fig. 16: Content of (A) starch and (B) ADPG in leaves of AGPase-deficient potato AGP62 and AGP85.  
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